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Kazuhisa HATAKEYAMA

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See MPEP chapter 600 concerning utility patent application contents.

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1. ☒ Fee Transmittal Form
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2. ☒ Specification [Total Pages -43]
(preferred arrangement set forth below)
 - Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☐ Drawing(s) (35 USC 113) [Total sheets -]
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6. Microfiche Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission
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 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
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18. CORRESPONDENCE ADDRESS

WENDEROTH, LIND & PONACK, L.L.P.
2033 "K" Street, N.W.
Suite 800
Washington, D.C. 20006
Phone:(202) 721-8200
Fax:(202) 721-8250

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SPECIFICATION

Method for Gene Analysis

5

Background of the InventionField of the Invention

The present invention relates to a method for gene analysis by hybridization, and more specifically, it relates to a novel method for gene analysis preferably utilized for gene analysis by hybridization using a DNA chip and the like, which method can be used for the analysis of nucleic acids, such as nucleotide sequence determination of nucleic acids, gene diagnosis of infectious diseases or genetic diseases, and monitoring of genome DNA expression.

Description of the Related Art

The analysis of genes by a hybridization technique utilizing a substrate (DNA chip or DNA array) having immobilized probe nucleic acids is widely utilized, for example, for nucleotide sequence determination, gene diagnosis of infectious and genetic diseases, monitoring expression of genome gene and so forth. For instance, SBH [Sequencing By Hybridization, R. Drmanac et al., Science, 260, 1649 (1993)], i.e., nucleotide sequencing via hybridization, is expected to be put into practical

use as a high-speed and low cost method. Further, the method for detecting mutations contained in genes using DNA chips [J.G. Hacia et al., Nature Genetics, 14,411-447(1996)] and the method of monitoring gene expression patterns using DNA chips [M. Schena et al., Science, 270,467-470(1995)] are drawing attention as methods enabling quick analysis of significant amounts of gene expression.

For these analyses of genes, an article called DNA chip or DNA array (DNA microarray or DNA macroarray) is utilized, which comprises nucleic acids such as DNA and/or RNA immobilized on a substrate.

As a substrate on which DNA is immobilized, membranes made of resin such as nylon membranes and polypropylene membranes, nitrocellulose membranes, glass plates or silicon plates are utilized. When detection of hybridization is performed by not using radioisotopes, but using, for example, fluorescent substances, it is preferable to utilize glass plates or silicon plates which contain no fluorescent substance.

However, when hybridization is performed on a DNA chip or DNA array (DNA microarray or DNA macroarray), the hybridization velocity is the major factor affecting the speed of gene analysis. The hybridization time required for gene analysis utilizing usual DNA chips or DNA arrays (DNA microarrays or DNA macroarrays) is 1 to 5 hours for gene sequence analysis [J.G. Hacia et al.,

Nature Genetics, 14, 441-447 (1996)], or 6 to 12 hours for gene sequence analysis [M. Shena et al., Proc. Natl. Acad. Sci. USA, Vol. 93, pp. 10614-10619 (1996)], and this has been a major problem in realizing gene analysis of higher speed utilizing the aforementioned method.

Summary of the Invention

The object of the present invention is to increase the speed of gene analysis by hybridization utilizing a probe nucleic acid.

The inventors of the present invention earnestly studied in order to achieve the aforementioned object. As a result, it was found that gene analysis speed can be increased by performing hybridization on a DNA chip or DNA array (DNA microarray or DNA macroarray), which consists of a substrate having an immobilized probe nucleic acid, in the presence of a double-stranded DNA-binding protein derived from a thermophilic bacterium. That is, it is considered that a double-stranded DNA-binding protein was bound to a double-stranded DNA in a DNA hybridization system in an equilibrated state to forward the reaction in a single direction (complementary double-stranded DNA forming direction), and that the heat resistance of the protein enabled the reaction at high temperatures and thereby realized higher speed and high-throughput of the gene analysis. The present invention has been accomplished on the basis

of these findings.

That is, the present invention provides a method for gene analysis comprising the step of detecting hybridization between a probe nucleic acid and a sample nucleic acid containing a target sequence that has a sequence complementary to that of the probe nucleic acid, wherein either the probe nucleic acid or the sample nucleic acid is immobilized on a substrate, at least one of the probe nucleic acid and the sample nucleic acid is DNA, and the hybridization is caused in the presence of a double-stranded DNA-binding protein.

The present invention also provides the aforementioned method for gene analysis wherein the sample nucleic acid is DNA.

The present invention further provides the aforementioned method for gene analysis wherein the double-stranded DNA-binding protein is derived from a hyperthermophilic bacterium.

The present invention further provides the aforementioned method for gene analysis wherein the double-stranded DNA-binding protein is derived from an archaebacterium.

The present invention further provides the aforementioned method for gene analysis wherein the double-stranded DNA-binding protein is derived from a bacterium belonging to the genus *Sulfolobus*.

The present invention further provides the

aforementioned method for gene analysis wherein the double-stranded DNA-binding protein is derived from *Sulfolobus solfataricus*.

The present invention further provides the
5 aforementioned method for gene analysis wherein the double-stranded DNA-binding protein is Sso7d protein derived from *Sulfolobus solfataricus*.

The present invention further provides the
aforementioned method for gene analysis wherein the
10 double-stranded DNA-binding protein is a protein having homology of 75% or more to the protein represented by the amino acid sequence of SEQ ID NO: 9.

The present invention further provides the
aforementioned method for gene analysis wherein the
15 sample nucleic acid is labeled.

The present invention further provides the
aforementioned method for gene analysis wherein amount
of the sample nucleic acid containing the target
sequence is analyzed based on intensity of hybridization
20 signal.

The present invention further provides the
aforementioned method for gene analysis wherein
detecting hybridization is performed by using a
plurality of probe nucleic acids and then polymorphism
25 in the target sequence is detected based on the result
of detection of hybridization.

The present invention further provides the

aforementioned method for gene analysis wherein detecting hybridization is performed by using a plurality of probe nucleic acids and then nucleotide sequence of the sample nucleic acid is determined based on the result of detection of hybridization.

The present invention further provides a test kit for detection of hybridization between a probe nucleic acid and a sample nucleic acid containing a target sequence that has a sequence complementary to that of the probe nucleic acid, which comprises at least a double-stranded DNA-binding protein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention will be explained in detail hereafter.

In the present invention, the term "double-stranded DNA-binding protein" refers to a protein which binds to chromosome of eucaryote or that of prokaryote strongly and concerns retention of higher-order structure of chromosome. That is, it comprises a protein having function to stabilize a complementary double-stranded DNA.

In the present invention, the term "sample nucleic acid" refers to a nucleic acid which is a subject of analysis such as nucleotide sequence determination or expression analysis, and it may be either DNA or RNA.

In the present invention, the term "probe nucleic

acid" refers to a nucleic acid which is utilized for detection of a target gene in a sample nucleic acid, and which may be either DNA or RNA. Examples of the probe nucleic acid include a probe containing an

5 oligonucleotide comprising usual base of nucleic acid that is A (adenine), T (thymine), G (guanine), C (cytosine) and U(uracil), and a probe consisting of a DNA fragment amplified by PCR and having a length of approximately 50-2,000 nucleotides. The length of the

10 probe nucleic acid is not particularly limited so long as it is a length hybridizable with the sample nucleic acid. Those having a length of 6-90 nucleotides, preferably 8-30 nucleotides, are usually used. However, nucleic acids of either longer or shorter than these

15 lengths may also be used.

As the probe nucleic acid, there may further be used a probe nucleic acid containing an oligonucleotide which comprises a modified nucleotide, for example, hypoxanthines such as inosine (Japanese Patent Laid-open

20 (Kokai) No. 8-70900(U.S. Patent No. 5,738,993)), 5-nitroindole, 3-nitropyrrole (Japanese Patent Laid-open (Kokai) No. 10-262675), 2-aminoadenine, 5-(1-propynyl)uracil (Tetrahedron Letters, Vol.33, p. 5307-5310) and so forth.

25 In the probe nucleic acid, as described in Japanese Patent Laid-open (Kokai) No. 8-70900 (U.S. Patent No. 5,738,993), a non-specific region may be

ligated to at least one of the ends of the sequence
region substantially complementary to the target
sequence in the sample nucleic acid. Ligation of such a
non-specific region enables increase of hybridization
5 sensitivity as well as facilitates differentiation
between a complementary hybrid and a hybrid having a
mismatch, in particular, end mismatch.

The non-specific region consists of at least one
nucleotide which has a base that can form a base pair
10 with a nucleotide constituting normal nucleic acids, but
is different from those of the nucleotides constituting
normal nucleic acids, or an oligomer thereof. Such a
base is preferably one that can associate with any of
bases constituting normal nucleic acid equally, and
15 strength of such association is preferably weaker than
that of the base pairs constituting a specific pairing.
As a specific example of such a base, hypoxanthine, 5-
nitroindole, 3-nitropyrrole and so forth can be
mentioned. As a specific example of such a nucleotide,
20 there can be mentioned deoxyinosine, which is a
deoxyribonucleotide having hypoxanthine as a base.

The number of nucleotides or oligomers thereof
constituting the non-specific region is not particularly
limited. However, it is preferably 2-20, more
25 preferably 2-8. The location for ligation of the non-
specific region to the specific region is not
particularly limited as well, and it may be either of

the 5' end and 3' end of the specific region. Both of the 5' end and 3' end of the specific region may be each ligated with a non-specific region. Among these cases, the latter case is particularly preferred. While the ratio of lengths of the specific region and the non-specific region may vary depending on the length of the specific region or the GC content, the length of the specific region is preferably equal to or longer than that of the non-specific region.

By providing a non-specific region in the probe nucleic acid as described above, the gene analysis can be performed with higher precision.

The aforementioned probe nucleic acid and the sample nucleic acid can easily be synthesized by using a commercially available DNA synthesizer.

The probe nucleic acid is utilized to detect a sample nucleic acid comprising a nucleotide sequence complementary to that of the probe nucleic acid. This complementary sequence to the probe nucleic acid is referred to as a "target sequence" in the present invention.

The method of the present invention is characterized in that the hybridization between a probe nucleic acid and a sample nucleic acid is performed in the presence of a double-stranded DNA-binding protein. The hybridization may be performed in the same manner as conventional methods for gene analysis by hybridization,

except for the use of the double-stranded DNA-binding protein. In addition, in the method of the present invention, at least one of the aforementioned probe nucleic acid or the sample nucleic acid should be DNA.

5 In the method of the present invention, it is preferable that the probe nucleic acid or the sample nucleic acid should be immobilized on a substrate, and the hybridization between the probe nucleic acid and a sample nucleic acid should be performed on the substrate.

10 In this case, it is preferable that the sample nucleic acid should be labeled to detect whether or not the hybridization has occurred. The method used for labeling is not particularly limited, and for example, it may include methods utilizing radioisotopes,

15 fluorescent dyes, biotin and so forth.

 As a substrate for hybridization, various kinds of materials, for example, membranes made of resin such as nylon membranes and nitrocellulose membranes, glass, silicon, etc are usually used. However, in the present

20 invention, the substrate is not limited to these materials, and any substrate can be utilized so long as DNA and RNA can be immobilized on it in some way.

 In this specification, the substrate on which a probe nucleic acid is immobilized is also referred to as

25 a "DNA chip" or a "DNA array (DNA microarray or DNA macroarray)" for convenience. However, as was described earlier, this does not mean that the nucleic acid to be

immobilized must be DNA, as it may also be RNA. Those comprising membranes such as nylon membrane, nitrocellulose membrane and so forth having an immobilized probe nucleic acid are generally referred to as "DNA (macro)array", and those comprising a rigid substrate composed of glass, silicon etc. having an immobilized probe nucleic acid are sometimes referred to as "DNA chip" or "DNA (micro)array". In the present invention, all of these may be used.

As the method for immobilization of the probe nucleic acid or the sample nucleic acid onto the substrate, there can be mentioned the method utilizing synthesis of nucleic acids directly onto a substrate [A.C. Pease et al., Proc. Natl. Acad. Sci. USA, 91, 5022-5026 (1994)], the methods comprising immobilization of synthesized nucleic acids [Z. Guo et al., Nucl. Acids Res., 22, 5456-5465 (1994)] or PCR products [M. Shena et al., Proc. Natl. Acad. Sci. USA, Vol. 93, pp. 10614-10619 (1996)] and so forth onto a substrate.

For example, the synthesis of a probe nucleic acid or a sample nucleic acid can be performed according to a standard protocol. In the synthesis, it is preferable to synthesize them with a cycle in which trityl groups, which are usually used as a protective group for the 5' end, are not removed, when the following purification method is used. The synthesized nucleic acid is preferably purified by using Poros Oligo R3 (produced by

PerSeptive Biosystems) or the like.

It is preferable to adjust the concentrations of the probe nucleic acid or the sample nucleic acid to predetermined concentrations prior to immobilization onto the substrate or hybridization thereof. For example, these nucleic acids are concentrated to dryness, and then suspended in a 0.5 M sodium hydrogencarbonate buffer (pH 8.4), TE buffer or the like, and they are quantified based on absorbance at 260 nm and adjusted to 1 nmol/ μ l.

Nucleic acids can be immobilized onto the substrate, for example, by bonding amino groups of amino-modified oligonucleotides to a nylon membrane having anionic carboxyl groups on its surface at a high density through amide bonds, as described below.

As for the substrate, nitrocellulose membranes, glass and so forth can also be utilized. It is particularly preferable to utilize glass when the sample DNA will be labeled with a fluorescent substance.

As the method for labeling and detection of nucleic acid, the RI method utilizing [γ - 32 P] ATP was used in the example described later, but either the fluorescent method or the biotin-avidin method may be used as well. Examples of the fluorescent substance include Cy3, Cy5, FITC (fluorescein isothiocyanate) and so forth. As for the labeling method, in addition to the 5' end labeling method, the random primer labeling

method, the nick translation method and so forth can be utilized as well.

Methods of the aforementioned synthesis of nucleic acid, hybridization, the labeling of nucleic acids and so forth are described in references well known to those skilled in the art, for example, Maniatis, T. et al., "Molecular Cloning, A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)) and so forth.

Although various kinds of double-stranded DNA-binding proteins are known as double-stranded DNA-binding proteins, it is preferable to utilize a thermostable double-stranded DNA-binding protein which is derived from a hyperthermophilic bacterium (bacterium which can grow at temperatures of 90°C or higher).

Hyperthermophilic bacteria are assumed to have some double-stranded DNA-binding protein because, when double-stranded DNA is separated from genome of hyperthermophilic bacteria and heated to 100°C, the DNA becomes single-stranded DNA.

Further, among hyperthermophilic bacteria, archaebacteria such as *Methanobacterium*, *Methanococcus*, *Archaeoglobus*, *Pyrococcus* and so forth contain double-stranded DNA-binding proteins such as histone-like proteins and HU protein.

Other than those, as double-stranded DNA-binding proteins derived from archaebacteria, there have been known proteins derived from *Sulfolobus* bacteria such as

Sso7d protein derived from *Sulfolobus solfataricus* [A. Guagliardi et al., J. Mol. Biol., Vol. 267, p.841-848 (1997)] ,Sac7d and Sac7e proteins derived from *Sulfolobus acidocaldarius* [J.G. McAfee et al., Biochemistry, Vol. 34, p. 10063-10077 (1995)], Sac7a and Sac7b proteins derived from *Sulfolobus acidocaldarius* [Kimura, M. et al., FEBS Letts., Vol. 176, p. 176-178(1984); Choli, T. et al., J. Biol. Chem., Vol.263, p.7087-7093(1988)] and so forth. However, it is more preferable to utilize the Sso7d protein derived from *Sulfolobus solfataricus*.

As shown in SEQ ID NO: 9, the Sso7d protein is a protein consisting of 63 amino acids [H. Baumann et al., Nature Structural Biology, Vol. 1, p. 808-819 (1994)]. In the present invention, a protein consisting of the 63 amino acids or a protein having a homology thereto of 75% or more in amino acid sequence are particularly preferably used as the double-stranded DNA-binding protein.

In the present invention, analysis of the homology is carried out by using the Lipman-Pearson method.

As software for analysis, "Genetyx" produced by Software Development Co., LTD. is used.

The result of analysis by using the method is shown as follows.

Sso7d vs. Sac7a : 84.5%

Sso7d vs. Sac7b : 87.5%

Sso7d vs. Sac7d : 84.5%

Sso7d vs. Sac7e : 83.3%

These double-stranded DNA-binding proteins have a
5 function of stabilizing the double strands
complementarily hybridized, and it is considered that
they can maintain the double strands acceleratedly
hybridized at high temperatures as it is without causing
re-dissociation thereof. It is considered that a
10 protein having such a function can be utilized in the
present invention like the specifically aforementioned
protein. Any special conditions are not particularly
required for the expression of the aforementioned
function of the double-stranded DNA-binding protein, and
15 ordinary hybridization conditions with the presence of
dithiothreitol(DTT), magnesium ions and so forth are
sufficient.

Further, the double-stranded DNA-binding protein
is preferably purified. The purification method may be
20 a conventional purification method for proteins, and for
example, purification can be performed by the method of
Bauman (Nature Structural Biology, Vol. 1, p.808-819
(1994)) and so forth.

Specifically, the purification of the Sso7d
25 protein, for example, can be performed by the following
procedure. First, the *Sulfolobus solfataricus* strain
DSM 1618 (strain IFO 15331) is cultured, and then the

obtained bacterial cells are disrupted by a French press or the like and centrifuged. The obtained supernatant fraction is fractionated by using a MonoQ (produced by Pharmacia) column, and the target fraction is

5 concentrated. The fraction is fractionated by using a Superose 6 column, and the target fraction is dialyzed and fractionated by using a MonoS column to perform the purification.

An example of purification of the Sso7d protein
10 obtained from *Sulfolobus solfataricus* is shown in the working examples mentioned hereinafter. It can also be purified from bacterial cells such as *Escherichia coli* cells in which the protein is highly produced thanks to a Sso7d protein gene introduced by a gene recombination
15 technique.

The hybridization can be performed in the same manner as the usual nucleic acid hybridization, except that the hybridization is performed in the presence of a double-stranded DNA-binding protein. Specifically the
20 hybridization can be performed as follows. First reducing agents such as dithiothreitol(DTT) and 2-mercaptoethanol, bovine serum albumin(BSA) and skim milk which prevent protein from non-specific binding to vessel and stabilize protein are added, and further protein
25 accessory factors such as magnesium chloride($MgCl_2$), salt-condensation regulators such as sodium chloride(NaCl) and potassium chloride(KCl) and so forth

are added as required into buffer such as Tris buffer, phosphate buffer, citric acid byffer, TES buffer, HEPES buffer or the like. The double-stranded DNA bidding protein is then added to the solution. In this

5 hybridization solution, the aforementioned oligonucleotide-immobilized nylon membrane (DNA (macro)array) and a labeled sample DNA are hybridized preferably for 1-20 minutes within a range of 40-120°C. When Sso7d is used as the double-staranded DNA binding

10 protein, to a Tris buffer, 0.1-100 mM of DTT, 0.1-100 mM of $MgCl_2$ and 1-100 $\mu g/\mu l$ of BSA (all of the concentrations are final concentrations) are added, and the Sso7d protein is added to the solution within a range of 0.001-10%. In this hybridization solution, the

15 oligonucleotide-immobilized nylon membrane (DNA (macro)array) and a labeled sample DNA are hybridized preferably for 1-15 minutes within a range of 40-70°C.

After the hybridization reaction, the membrane was washed with buffer such as Tris buffer, phosphate buffer,

20 citric acid byffer, TES buffer, HEPES buffer or the like for 1-10 minutes within a range of 10-50°C, and then dried. In this case, adding a small amount of surfactant such as sodium dodecyl sulfate (SDS) is preferable because it can prevent from non-specific

25 binding and step down backgraound. When Sso7d is used as the double-staranded DNA binding protein, after the hybridization reaction, it is preferable that the

membrane should be washed with citric acid buffer such as SSC, more preferably buffer added with SDS within a range of 0.001-0.05% as required to SSC, for 3-10 minutes within a range of 10-40 °C, and then air-dried.

- 5 As for the hybridization signal, radiation dose or the like of each dot on the dried nylon membrane can be measured by autoradiography etc. to calculate the hybridization strength.

Detection of hybridization can be measured by a method suitable for each of various labeling methods. In the gene expression monitoring method, it is preferable to use fluorescent labeling because this method enables simultaneous detection of the expression strength for a plurality of sample nucleic acids by labeling them with a plurality of fluorescent dyes each having a different detection wavelength.

Examples of the application of the nucleotide sequence analysis according to the present invention include: DNA nucleotide sequence determination [Genomics, Vol. 4, p. 114-128 (1989)], diagnosis of infectious and genetic diseases etc. [J.G. Hacia et al., Nature Genetics, 14, 441-447 (1996)], mapping of giant genome DNA [BioTechniques, vol.17, No.2, p.328-336 (1994)], single-nucleotide polymorphisms (SNPs) [Wang et al. Science, Vol. 280, p. 1077-1082 (1998)], the amplification of genes or analysis of deleted regions by

CGH (comparative genomic hybridization) method [D. Pinkel et al. Nat. Genet. Vol. 20, p. 207-211 (1998)], gene expression monitoring [M. Shena et al., Proc. Natl. Acad. Sci. USA, Vol. 93, pp.10614-10619 (1996)] and so
5 forth.

As a diagnostic method for infectious diseases, there can be mentioned, for example, a method of detecting presence of a causative factor by extracting DNA from subject's blood etc., preparing a DNA probe
10 based on a sequence specific to each of various pathogens [A. Troesch et al., J. Clin. Microbiol., Vol.37, p.49-55(1999)], and performing hybridization reaction according to the present invention to perform gene analysis for the extracted DNA.

15 As a diagnostic method for genetic diseases, there can be mentioned, for example, a method of detecting presence or absence of a mutation in a gene by preparing an oligonucleotide based on a sequence specific to a causative gene of genetic disease [M. J. Kozal et al.,
20 Nature Medicine, Vol.2, p.753-759(1996)], and performing hybridization of the oligonucleotide with chromosomal DNA obtained from a subject according to the present invention to perform gene analysis.

The giant genome DNA mapping is an essential
25 technique for the genome DNA analysis project and so forth. By performing hybridization of many DNA probes prepared by the method of the present invention with

genes of a genome bank, the location of each clone on the genome can be determined.

Further, a test kit for using to performe the
aforementioned gene analysis can be prepared by using a
5 double-stranded DNA-binding protein. Such a test kit is
constituted by components similar to those of ordinary
test kits for gene analysis utilizing hybridization
except for the use of the double-stranded DNA-binding
protein. That is, the test kit of the present invention
10 comprises at least a double-stranded DNA-binding protein
and, as optional components, washing solution, diluent,
hybridization solution and so forth.

EXAMPLES

15

The present invention will be explained more
specifically with reference to the following examples.
However, the present invention is no way limited by
these examples.

20

Example 1

(A) Synthesis of probe DNA and sample DNA

The oligonucleotides shown in Table 1 were
synthesized by using a DNA synthesizer (apparatus name:
25 Expedite 8909) manufactured by PerSeptive Biosystems.

Immobilization of the oligonucleotides may be
facilitated by modifying their 5' or 3' ends with 5'

amino-modified C6 (produced by Glen Research) or the like. The oligonucleotide numbers used below corresponds to SEQ ID NOS in SEQUENCE LISTING.

The oligonucleotide (3) is a sample DNA to be analyzed, the oligonucleotide (1) is a DNA probe completely complementary to the oligonucleotide (3) (completely matched), and the oligonucleotide (2) is a DNA probe complementary to the oligonucleotide (3), but containing one nucleotide mismatch in the internal region.

Table 1

No.	Nucleotide sequence	Note
15	(1) 5'-XATGTAAGTCGCCTT-3'	Completely matched probe
	(2) 5'-XATGTAACCCGCCTT-3'	One base-mismatched probe
	(3) 5'-CCAACGATCAAGGCGAGTTACATGATCC-3'	Sample DNA

In the table, X represents 5' amino-modified C6.

The synthesis of the aforementioned oligonucleotides and the sample DNA were performed in accordance with a standard protocol (Nucleic Acid Synthesis System User's Guide, PerSeptive Biosystems) by using a cycle in which the trityl groups were not removed, which were the protective groups of the 5' ends. The synthesized DNAs were purified by using Poros Oligo R3 (produced by PerSeptive Biosystems).

The oligonucleotides (1) and (2), and the sample DNA (3) were concentrated to dryness, and then suspended in a 0.5 M sodium hydrogencarbonate buffer (pH 8.4) for (1) and (2), or TE buffer for (3), and they are
5 quantified based on absorbance at 260 nm and adjusted to 1 nmol/ μ l.

(B) Immobilization of probe DNA (preparation of DNA array)

10 Immobilization of the oligonucleotides on a substrate was attained by bonding amino groups of the amino-modified oligonucleotides to a nylon membrane containing anionic carboxyl groups on its surface at a high density through amide bonds, as described below.
15 A Biodyne C (trade mark, produced by Pall) membrane was rinsed with 0.1 N HCl to acidify it, and immersed in 20% EDC (1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride) at room temperature for 15-30 minutes. The membrane was lightly
20 rinsed with deionized water and 0.5 M sodium hydrogencarbonate buffer (pH 8.4), then mounted on a dot blot apparatus (produced by Bio-Rad) and allowed to react with the amino-modified oligonucleotide (1) or (2), which was suspended in a 0.5 M sodium hydrogencarbonate
25 buffer (pH 8.4), at room temperature for 15 minutes.

The membrane was washed with TBS (Tris-buffered saline)/0.1% Tween-20, then treated with 0.1 N NaOH for

10 minutes, lightly rinsed with deionized water, and air-dried.

(C) Labeling of sample DNA

5 As for the labeling of the sample DNA, the 5' end was radioactively-labeled with [γ - ^{32}P] ATP. The reaction was performed by using a DNA 5' end labeling kit (MEGALABEL, produced by Takara Shuzo).

10 (D) Purification of Sso7d protein

 Cultivation was performed at 75°C under an aerobic condition in a medium (1 g of yeast extract, 2.3 g of $(\text{NH}_4)_2\text{SO}_4$, 0.08 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.28 g of KH_2PO_4 , 0.3 g of $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$, 0.03 g of
15 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L of distilled water, adjusted to pH 3.0 with sulfuric acid) added with 10 g/L of saccharose, using a membrane fermenter. The cells were given with heat shock at 88°C for 90 minutes, and harvested by centrifugation.

20 The cells (100 g) were dissolved in Buffer A (10 mM Tris buffer, pH 8.8, 20 mM NaCl, 10% glycerol), and then disrupted with a French press. The disrupted cell suspension was centrifuged, and the obtained supernatant was dialyzed against Buffer A. Then, the dialysate was
25 applied to a MonoQ column (produced by Pharmacia) and equilibrated with Buffer A. The Sso7d protein was obtained in the outflow fraction. This fraction was

concentrated with Amicon, applied to a Superose 6 column and equilibrated with Buffer B (30 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl). The fractions containing Sso7d were collected and dialyzed against Buffer C (50 mM potassium phosphate, pH 6.0, 50 mM NaCl), and then applied to a MonoS column (produced by Pharmacia). The column was equilibrated with Buffer C and eluted with a gradient using Buffer D (50 mM potassium phosphate, pH 8.0, 1 M NaCl) to obtain the Sso7d protein at a 25% concentration of Buffer D.

(E) Hybridization reaction

(a) Hybridization strength

70 $\mu\text{g/ml}$ of Sso7d protein was added to Buffer E (20 mM Tris buffer, pH 7.5, 2 mM DTT, 5 mM MgCl_2 , 10 $\mu\text{g}/\mu\text{l}$ of BSA), and the aforementioned oligonucleotide-immobilized nylon membrane and the radioactively-labeled sample DNA were allowed to hybridize at 60°C for 3 minutes in the buffer. As a control, hybridization reaction was also performed in the same manner without adding the Sso7d protein.

After the hybridization reaction, the membrane was washed with 1 x SSC/0.03% SDS buffer at 30°C for 5 minutes and air-dried. The hybridization signal was evaluated by measuring radiation dose of each dot on the air-dried nylon membrane by autoradiography to calculate hybridization signal strength. The results are shown in

Table 2. The hybridization signal strength is represented with relative values based on the hybridization signal strength of the probe oligonucleotide (1) when Sso7d was added, which is taken as 100.

Table 2

No.	Nucleotide sequence	Addition of Sso7d	Hybridization
			signal strength
10	(1) 5'-XATGTAACCTCGCCTT-3' Completely matched	Not added	<5
	(2) 5'-XATGTAACCCGCCTT-3' Mismatched	Not added	<5
15	(1) 5'-XATGTAACCTCGCCTT-3' Completely matched	Added	100
	(2) 5'-XATGTAACCCGCCTT-3' Mismatched	Added	10

In the table, X represents 5' amino-modified C6.

In the hybridization systems not added with Sso7d, hybridization between the probe DNA immobilized on the substrate (on the DNA array) and the sample DNA was not substantially detected for both of the completely matched probe (1) and the mismatched probe (2).

On the other hand, when the Sso7d protein was added to the hybridization systems, only the completely matched probe hybridized with the sample DNA on the DNA array. In comparison with the hybridization signal

strength of the completely matched probe, the hybridization signal strength of the mismatch probe was considerably weaker, and these were clearly distinguishable.

5

(b) Change of hybridization signal strength with change of Sso7d protein concentration

The Sso7d protein was added to Buffer E at a concentration of 70 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ or 10 $\mu\text{g/ml}$, and the nylon membrane on which the completely matched probe in Table 1, the probe oligonucleotide (1), was immobilized, and the radioactively-labeled sample DNA were hybridized at 60°C for 3 minutes in each solution.

After the hybridization reaction, the membrane was washed with a solution of 1 x SSC/0.03% SDS buffer at 30°C for 5 minutes, and then air-dried. The hybridization signal was evaluated by measuring radiation dose of each dot on the air-dried nylon membrane by autoradiography to calculate hybridization signal strength. The results are shown in Table 3.

In the table, the hybridization signal strength is represented with relative values based on the hybridization signal strength obtained when Sso7d was added at a concentration of 70 $\mu\text{g/ml}$, which is taken as 100.

Table 3

No. Addition of Sso7d		Hybridization signal Strength
5	(1) 70 $\mu\text{g/ml}$	100
	(2) 50 $\mu\text{g/ml}$	100
	(3) 25 $\mu\text{g/ml}$	40
	(4) 10 $\mu\text{g/ml}$	10

The completely matched probe strongly hybridized to the sample DNA on the DNA array at a Sso7d protein concentration of 50 $\mu\text{g/ml}$ or more. However, at the concentrations of 25 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, the hybridization signal strength was weak. These results show that the hybridization signal sensitivity was increased by addition of Sso7d.

(c) Change of hybridization signal strength with hybridization reaction time

A nylon membrane on which the completely matched probe, the oligonucleotide (1), was immobilized, and a radioactively-labeled sample DNA were allowed to hybridize at 60°C under the same condition as the above (E) (a), except that the hybridization reaction time was changed. The hybridization signal strength was measured at 3, 10 and 30 minutes after the start of the hybridization.

The hybridization signal strength did not vary

with the reaction time. The hybridization had been almost fully completed within 3 minutes of reaction time, thus showing that the hybridization reaction speed was increased with the addition of the Sso7d protein.

5

Example 2

(A) Synthesis of probe DNA and sample DNA

The oligonucleotides shown in Table 4 were synthesized by using a DNA synthesizer (apparatus name: Expedite 8909) manufactured by PerSeptive Biosystems. Immobilization of the oligonucleotides may be facilitated by modifying their 5' or 3' ends with 3' amino-modified C6 (produced by Glen Research) or the like. As for the end of the sample DNA, its 5' end may be labeled with fluorescence using Cy5 Amidite (produced by Amersham Pharmacia Biotech). The oligonucleotide numbers used below corresponds to SEQ ID NOS in SEQUENCE LISTING.

The oligonucleotide (8) is a sample DNA to be analyzed, the oligonucleotides (4) and (6) are DNA probes completely complementary to the oligonucleotide (8) (completely matched), and the oligonucleotides (5) and (7) are DNA probes complementary to the oligonucleotide (8), but containing one nucleotide mismatch in the intermediary region.

Table 4

No.	Nucleotide sequences	Note
	(4) 5'-ATCGCCCGGACTCX-3'	Completely matched probe
5	(5) 5'-ATCGCCTGGACTCX-3'	One base mismatched probe
	(6) 5'-iiiiTCGCCCGGACTiiiiX-3'	Completely matched probe
	(7) 5'-iiiiTCGCCTGGACTiiiiX-3'	One base mismatched probe
	(8) 5'-Cy5AGTCTCGGAGTCCGGGCGATGGCCAC-3'	Sample DNA

In the table, i represents inosine residue, X represents
 10 3' amino-modified C6, and Cy5 represents a fluorescent
 label by Cy5.

The syntheses of the aforementioned
 oligonucleotides and the sample DNA were performed in
 15 accordance with a standard protocol (Nucleic Acid
 Synthesis System User's Guide, PerSeptive Biosystems) by
 using a cycle in which the trityl groups were not
 removed, which were the protective groups of the 5' ends.
 The synthesized DNA were purified by using Poros Oligo
 20 R3 (produced by PerSeptive Biosystems).

The oligonucleotides (4) to (8) and the sample DNA
 were concentrated to dryness, and then suspended in a
 0.5 M sodium hydrogencarbonate buffer (pH 8.4) for (4)
 to (7), or TE buffer for (8), and they are quantified
 25 based on absorbance at 260 nm and adjusted to 100
 pmol/ μ l.

(B) Immobilization of probe DNA (preparation of DNA microarray or DNA chip)

Immobilization of the oligonucleotides was performed as follows. First, solutions of the

5 oligonucleotide probe nucleic acids (4) to (7) were each spotted onto a Silylateds Slide of TeleChem (slide glass having aldehyde groups on its surface) using a GTMASS Stamping apparatus produced by Nippon Laser & Electronics Lab. Then, according to the protocol of

10 TeleChem, the nucleic acid was bonded on the slide glass through terminal covalent bonds (a Schiff base was formed by amino group and aldehyde group) as follows. First, the slide glass was mounted on a slide rack, and washed twice with 0.2% SDS at 25°C for 2 minutes with

15 sufficient stirring in a beaker. Then, it was washed twice with sterilized water at 25°C for 2 minutes with sufficient stirring, and further treated with sterilized water at 98°C for 2 minutes. The slide glass was air-dried at room temperature for 5 minutes, then

20 transferred into a sodium hydrogenborate solution [prepared by dissolving 1 g of NaBH_4 in 300 ml of PBS buffer (prepared by dissolving 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogenphosphate, and 0.24 g of potassium

25 dihydrogenphosphate in deionized water, adjusting it to pH 7.4 with hydrochloric acid, and filling it up to 1000 ml) and 100 ml of ethanol], treated in the solution at

25°C for 5 minutes, washed three times with 0.2% SDS at 25°C (room temperature) for 1 minute, finally washed with sterilized water at 25°C for 1 minute, and air-dried.

5

(C) Hybridization reaction

70 µg/ml of the Sso7d protein and 100 pmol/ml of the sample DNA (8) were added to Buffer E (20 mM Tris buffer, pH 7.5, 2 mM DTT, 5 mM MgCl₂, 10 µg/µl of BSA), and the aforementioned oligonucleotide-immobilized slide glass (DNA microarray) and the fluorescently labeled sample DNA were allowed to hybridize at 60°C for 6 minutes in the solution. As a control, hybridization reaction was also performed in the same manner without adding the Sso7d protein.

After the hybridization reaction, the membrane was washed with 1 x SSC/0.03% SDS buffer at 25°C for 5 minutes, rinsed with 0.2 x SSC, and then further rinsed with 0.5 x SSC. After the washing solution was removed by centrifugation, the slide glass was air-dried.

The hybridization signal was evaluated by measuring amount of fluorescent dye of each spot on the air-dried slide glass by using ScanArray 3000 produced by Genaral Scanning to calculate the hybridization signal strength. The results are shown in Table 5. The results are represented with relative values based on the hybridization signal strength of the probe DNA (4)

obtained with the addition of Sso7d, which is taken as 100.

In the hybridization systems not added with Sso7d, hybridization between the probe DNA immobilized on the substrate (on the DNA array) and the sample DNA was not substantially detected under the aforementioned
5 conditions.

On the other hand, when the Sso7d protein was added to the hybridization systems, only the completely matched probes hybridized with the sample DNA on the DNA array. In comparison with the hybridization signal
10 strength of the completely matched probes, the hybridization signal strength of the mismatched probes was considerably weaker, and these were clearly distinguishable.

15 According to the present invention, gene analysis by hybridization utilizing a probe nucleic acid can be quickly performed with high precision and high sensitivity.

20 Having thus described the present invention, it will be obvious that several aspects of the invention may be modified in various ways. Such variations are not to be regarded as departures from the spirit and scope of the invention, and all such modifications would
25 be obvious to those skilled in the arts, and are intended to be included within the scope of the following claims.

SEQUENCE LISTING

<110> Kazuhisa HATAKEYAMA

<120> Method for Gene Analysis

<150> JP 11-144749

<151> 1999-05-25

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<213> Sulfolobus Solfataricus

<300>

<301> Herbert Baumann, Stefan Knapp, Thomas Lundback, Rudolf Ladenstein and Torleif Hard

<302> Solution structure and DNA-binding properties of a thermostable protein from the archaeon Sulfolobus Solfataricus

<303> Nature structural biology

<304> 1

<305> 11

<306> 808-819

<307> 1994-11-1

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			20					25					30		
Tyr	Asp	Glu	Gly	Gly	Gly	Lys	Thr	Gly	Arg	Gly	Ala	Val	Ser	Glu	Lys
		35					40					45			
Asp	Ala	Pro	Lys	Glu	Leu	Leu	Gln	Met	Leu	Glu	Lys	Gln	Lys	Lys	
	50						55				60				

WHAT IS CLAIMED IS:

1. A method for gene analysis comprising the step
of detecting hybridization between a probe nucleic acid
5 and a sample nucleic acid containing a target sequence
that has a sequence complementary to that of the probe
nucleic acid, wherein either the probe nucleic acid or
the sample nucleic acid is immobilized on a substrate,
at least one of the probe nucleic acid and the sample
10 nucleic acid is DNA, and the hybridization is caused in
the presence of a double-stranded DNA-binding protein.

2. The method according to claim 1, wherein the
sample nucleic acid is DNA.

15

3. The method according to claim 1, wherein the
double-stranded DNA-binding protein is derived from a
hyperthermophilic bacterium.

20 4. The method according to claim 1, wherein the
double-stranded DNA-binding protein is derived from an
archaebacterium.

5. The method according to claim 1, wherein the
25 double-stranded DNA-binding protein is derived from a
bacterium belonging to the genus *Sulfolobus*.

6. The method according to claim 1, wherein the double-stranded DNA-binding protein is derived from *Sulfolobus solfataricus*.

5 7. The method according to claim 1, wherein the double-stranded DNA-binding protein is Sso7d protein derived from *Sulfolobus solfataricus*.

8. The method according to claim 1, wherein the
10 double-stranded DNA-binding protein is a protein having homology of 75% or more to the protein represented by the amino acid sequence of SEQ ID NO: 9.

9. The method according to claim 1, wherein the
15 sample nucleic acid is labeled.

10. The method according to claim 1, wherein
amount of the sample nucleic acid containing the target
sequence is analyzed based on intensity of hybridization
20 signal.

11. The method according to claim 1, wherein
detecting hybridization is performed by using a
plurality of probe nucleic acids and then polymorphism
25 in the target sequence is detected based on the result
of detection of hybridization.

12. The method according to claim 1, wherein
detecting hybridization is performed by using a
plurality of probe nucleic acids and then nucleotide
sequence of the sample nucleic acid is determined based
5 on the result of detection of hybridization.

13. A test kit for detection of hybridization
between a probe nucleic acid and a sample nucleic acid
containing a target sequence that has a sequence
10 complementary to that of the probe nucleic acid, which
comprises at least a double-stranded DNA-binding protein.

ABSTRACT OF THE DISCLOSURE

In a method for gene analysis comprising the step of detecting hybridization between a probe nucleic acid and a sample nucleic acid containing a target sequence that has a sequence complementary to that of the probe nucleic acid, the hybridization is caused on a substrate on which either the probe nucleic acid or the sample nucleic acid is immobilized, in the presence of a double-stranded DNA-binding protein to improve analysis speed of a method for gene analysis by hybridization using a probe nucleic acid.

DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

(X) Original () Supplemental () Substitute () PCT () Design

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: METHOD FOR GENE ANALYSIS

of which is described and claimed in:

(X) the attached specification, or

() the specification in the application Serial No. _____ filed _____;
and with amendments through _____ (if applicable), or

() the specification in International Application No. PCT/ _____, filed _____, and as amended on _____ (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
Japan	11-144749	May 25, 1999	YES

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint John T. Miller, Reg. No. 21,120; Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Jeffrey Nolton, Reg. No. 25,408; Warren M. Cheek, Jr., Reg. No. 33,367; Nils E. Pedersen, Reg. No. 33,145 and Charles R. Watts, Reg. No. 33,142, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., attorneys to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from _____ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

Send Correspondence to

WENDEROTH, LIND & PONACK, L.L.P.
2033 K Street, N.W., Suite 800
Washington, DC 20006

Direct Telephone Calls to:

WENDEROTH, LIND & PONACK, L.L.P.
Area Code (202) 721-8200

Direct Facsimile Messages to:

Area Code (202) 721-8250

Full Name of First Inventor	FAMILY NAME HATAKEYAMA	FIRST GIVEN NAME KAZUHISA	SECOND GIVEN NAME
Residence & Citizenship	CITY Inashiki	STATE OR COUNTRY Japan	COUNTRY OF CITIZENSHIP Japan
Post Office Address	ADDRESS c/o Mitsubishi Chemical Corporation, Tsukuba Research Center, 300-0332, 8-3-1, Chuo, Ami, Inashiki, Ibaraki, Japan		
Full Name of Second Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
Full Name of Third Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
Full Name of Fourth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Fifth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Sixth Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

Full Name of Seventh Inventor	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
Residence & Citizenship	CITY	STATE OR COUNTRY	COUNTRY OF CITIZENSHIP
Post Office Address	ADDRESS	CITY	STATE OR COUNTRY ZIP CODE

I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor Kazuhisa Hatakeyama Date May 11, 2000
Kazuhisa HATAKEYAMA

2nd Inventor _____ Date _____

3rd Inventor _____ Date _____

4th Inventor _____ Date _____

5th Inventor _____ Date _____

The above application may be more particularly identified as follows:

U.S. Application Serial No. _____ Filing Date _____

Applicant Reference Number _____ Atty Docket No. _____

Title of Invention _____